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## Preparation of isotopically labelled recombinant $\beta$ -defensin for NMR studies\*\*

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### Abbreviations:

AMPs, antimicrobial peptides; HBD2, human  $\beta$ -defensin 2; TAP, tracheal antimicrobial peptide; LAP, lingual antimicrobial HBD1, peptide human  $\beta$ -defensin; LPS, lipopolysaccharides; KSI, ketosteroid isomerase; HEK, human embryonic kidney; GuHCl, guanidine hydrochloride; IPTG, isopropyl- $\beta$ -d-1-thiogalactopyranoside

### Keywords:

Defensin; Antimicrobial peptide; NMR; <sup>15</sup>N; Mass spectrometry; Isotopic label

## Abstract

$\beta$ -Defensins are a family of cationic peptides that contain six invariant cysteine residues that form characteristic disulfide bonds between Cys<sup>1</sup>-Cys<sup>5</sup>, Cys<sup>2</sup>-Cys<sup>4</sup> and Cys<sup>3</sup>-Cys<sup>6</sup>. They have been shown to act as potent antimicrobial agents and chemokines. Human  $\beta$ -defensin 2 (HBD2) was first isolated from psoriatic skin lesions and the structure of synthetic material has been solved by X-ray crystallography and NMR spectroscopy both of which are consistent with a fold that contains an Nterminal  $\alpha$ -helix and three antiparallel  $\beta$ -strands. Here, we report the expression and purification of the first isotopically labelled  $\beta$ -defensin (<sup>15</sup>N HBD2) with 100 % incorporation of <sup>15</sup>N using a recombinant *E. coli* method. Multidimensional NMR spectroscopy experiments: 2D <sup>1</sup>H-<sup>15</sup>N HSQC, 3D HSQC-TOCSY and 3D HSQCNOESY allows for the assignment of resonances with no overlapping or ambiguous peaks. This isotopically-labelled peptide is highly suitable for studying the interactions between HBD2 and a range of components from both the mammalian immune system and bacterial pathogens.

## Introduction

Defensins are cationic, cysteine-rich antimicrobial peptides (AMPs) that are active in low concentrations against a range of pathogenic organisms. Initially characterised as bactericidal agents isolated from various tissues (e.g. skin, lung), interest in mammalian defensins has increased recently because of their expanding roles in innate and adaptive immunity [1-4]. Depending on the spacing between the cysteine residues and the arrangement of their disulfide bonds, they are classified into three categories:  $\alpha$ ,  $\beta$  and  $\theta$ -defensins. The  $\alpha$ -defensins form disulfide bonds between Cys<sup>1</sup>-Cys<sup>6</sup>, Cys<sup>2</sup>-Cys<sup>4</sup> and Cys<sup>3</sup>-Cys<sup>5</sup> whereas  $\beta$ -defensins form disulfide bonds between Cys<sup>1</sup>-Cys<sup>5</sup>, Cys<sup>2</sup>-Cys<sup>4</sup> and Cys<sup>3</sup>-Cys<sup>6</sup>. Less common are the  $\theta$ -defensins which are cyclic peptides first isolated from the leukocytes of primates [5]. A homologous peptide with antiviral properties, encoded by a pseudogene, was later found to be expressed in human bone marrow [6].

Following on from the initial discovery of the  $\alpha$ -defensins [1], the first reported  $\beta$ -defensin was isolated from extracts of the bovine tracheal mucosa and referred to as tracheal antimicrobial peptide (TAP) [7]. This finding was followed by the isolation of bovine neutrophil  $\beta$ -defensin (BNBD-12) [8] and bovine lingual antimicrobial peptide (LAP) [9]. The first human  $\beta$ -defensin (HBD1) was extracted from the blood of renal patients [10], and subsequently the isolation of human  $\beta$ -defensin 2 (HBD2) and human  $\beta$ -defensin 3 (HBD3) from the skin of patients with psoriasis were reported [11-13]. Now, more than a few dozen human  $\beta$ -defensins have been identified in various epithelial tissue such as that of the respiratory tract [14], gastrointestinal tract [15], oral cavity [16] and reproductive system [17] with the first three being the most extensively studied.

Presently, there are only a limited number of high resolution structures of  $\beta$ -defensins reported. The first solved structure was over a decade ago for BNBD-12 isolated from bovine neutrophils using NMR spectroscopy [18]. Since then, the structures of synthetic and recombinant forms of human  $\beta$ -defensins; HBD1 [19], HBD2 [20-22], HBD3 [23], murine  $\beta$ -defensins; mBD7, mBD8 [20], and an avian  $\beta$ -defensin isolated from penguin stomach, Sphe-2 [24] have all been solved by NMR spectroscopy and/or x-ray crystallography. For HBD2, both the crystal and solution structures were solved and showed similar characteristics; however, the structure determined by x-ray crystallography displayed evidence of higher order oligomerization [21]. In contrast, the solution structure determined by NMR spectroscopy found the HBD2 to exist as a monomer [22]. The difference in aggregation states was attributed to differences in experimental conditions and peptide concentrations. However, solution structures of HBD3 showed signs of dimerization even at low concentrations, which may help explain its increased antimicrobial activity compared to HBD1 and HBD2 [23].

Although  $\beta$ -defensins possess very low sequence homology with only eight conserved residues, their tertiary structures are remarkably similar. They are composed of three  $\beta$ -strands that are arranged in an antiparallel fashion, and most contain an N-terminal  $\alpha$ -helix. Furthermore, all  $\beta$ -defensins have a bulge in the second strand involving the highly conserved Gly residue. Typically, the charged residues form one surface and the non-polar residues form a hydrophobic surface, but the extent of hydrophobicity is different from one  $\beta$ -defensin to another [4]. In all cases, the amphiphilic nature of the peptide suggests that the charged residues interact with the bacterial membranes through electrostatic interactions and the hydrophobic residues disrupt the cell wall by interacting with the lipid bilayer [25, 26]. Although structurally similar, slight variations substantially influence their activity. HBD3 is effective against both Gram positive and negative bacteria and is less salt sensitive than HBD1 and HBD2 whereas the latter peptides are less cationic and only effective against Gram-negative bacteria [23].  $\beta$ -defensins have also demonstrated chemotactic activities as they can attract immature dendritic cells and memory T-cells [27]. Each of the peptides HBD1-3 have been shown to act through the chemokine GPCR receptor CCR6 [27, 28]. Therefore, it is not surprising that these structures are similar to MIP-3 $\alpha$ , the natural chemokine of CCR6, which also contains antiparallel triple  $\beta$ -strands [4]. In one study, the structural and functional properties of 26 single-site mutants of HBD1 were analyzed and revealed that the cationic residues at the C-terminus are important for antimicrobial activity and that the residues in the N-terminal  $\alpha$ -helical region are essential for chemotactic activity with CCR6 transfected cells [28]. Recently, our own group has explored the structure/activity relationship of a mouse defensin, Defb14 and found that a linear peptide derivative, with no S-S bonds could act as a potent chemokine [29].

In order to better understand how defensins function at a molecular level, it is important to look at binding with various biologically-relevant ligands. Recently, the interactions of HBD2 and HBD3 with model membranes were investigated using circular dichroism, transmission or reflection infrared spectroscopy and dye release [30]. In addition, the interactions of the carboxy-terminal regions of HBD1-3 were examined with

phospholipids at the air-water interface and inner membrane of *E. coli* [31]. Evidence exists of other AMPs interacting with sugars [32] and lipopolysaccharides (LPS) [33, 34] and these ligands should be used to examine their interactions with  $\beta$ -defensins. Analysis and elucidation of such HBD2/ligand complexes are complicated so the introduction of isotopic labels into the peptides can act as tools to facilitate such studies.

We recently reported an efficient procedure for the isolation of recombinant, unlabelled HBD2 in *E. coli* using a ketosteroid isomerase (KSI) fusion partner [35]. The HBD2 produced by this method displayed antimicrobial activity against a range of microbes and was chemotactic for human embryonic kidney (HEK) cells expressing CCR6. Here, we report the preparation of the first isotopically labelled defensin,  $^{15}\text{N}$  HBD2 using optimized growth conditions. The high-resolution mass spectrum revealed 100 % incorporation of the  $^{15}\text{N}$  isotope. The introduction of this label allowed us to perform multidimensional NMR experiments making it possible to resolve overlapping signals and assign unambiguously the amide resonances of all the residues in the peptide sequence. These assignments serve as convenient probes for studying defensin/ligand interactions.

## Materials and methods

### MATERIALS

Chemicals used for the 2 x YT and minimal media were purchased from Sigma- Aldrich and Fisher Scientific. The guanidine hydrochloride (GuHCl),  $^{15}\text{N}$  labelled ammonium sulfate and  $^{15}\text{N}$ -ISOGRO™ were purchased from Sigma-Aldrich and the HPLC grade solvents were from VWR International. The His-KSI-HBD2 plasmid was prepared and used as described [35].

### PROTEIN EXPRESSION

The *Escherichia coli* strain BL21 (DE3) containing the plasmid pET-28a/His-KSIHBD2 [35] was grown in 6 x 6 mL of 2 x YT medium containing 30  $\mu\text{g}/\text{mL}$  kanamycin at 37 °C. Once the OD600 reached 0.6-0.8, cells were centrifuged (3000 rpm for 10 minutes, 4 °C) and the supernatant was removed. The pellet was washed and resuspended in M9 minimal medium three times, and the combined suspensions were added to 3 L fortified M9 minimal medium with 30  $\mu\text{g}/\text{mL}$  kanamycin. The M9 minimal medium consisted of 6.8 g  $\text{Na}_2\text{HPO}_4$ , 3 g  $\text{KH}_2\text{PO}_4$  and 0.5 NaCl in 3L deionized water, with pH adjusted to 7.4. The fortified minimal medium was made up with the addition of 2 mM  $\text{MgSO}_5$ , 0.10 mM  $\text{CaCl}_2$ , 0.12 mM thiamine, 0.16 mM biotin, 45 mL 20 % glucose, 7.6 mM ( $^{15}\text{NH}_4$ ) $_2\text{SO}_4$ , 0.5 g/L  $^{15}\text{N}$ -ISOGRO™ and trace metal solution [36] with concentrations of 50  $\mu\text{M}$   $\text{FeCl}_3$ , 20  $\mu\text{M}$   $\text{CaCl}_2$ , 10  $\mu\text{M}$   $\text{MnCl}_2$ , 10  $\mu\text{M}$   $\text{ZnSO}_4$ , 2  $\mu\text{M}$   $\text{CoCl}_2$ , 2  $\mu\text{M}$   $\text{CuSO}_4$ , 2  $\mu\text{M}$   $\text{NiCl}_2$ , 2  $\mu\text{M}$   $\text{NaMoO}_4$ , 2  $\mu\text{M}$   $\text{Na}_2\text{SeO}_3$ , 2  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ . The summary of the components used in the fortified

minimal medium is presented in Table 1. The 3 L of culture was divided into 6 x 1 L flasks with approximately 500 mL in each and shaken at 30 °C. When the OD<sub>600</sub> reached 0.5, protein expression was induced by 1 mM isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) and the cells were further incubated for 6 h. Cells were isolated by centrifugation (5500 rpm for 12 minutes, 4 °C). From 3 L of culture, approximately 7 g of wet weight *E. coli* paste was obtained.

**Table 1.** Fortified M9 Minimal Medium at pH 7.4.

<b>M9 Medium</b>	Final Concentration in 3L Total Volume
Na <sub>2</sub> HPO <sub>4</sub>	16 mM
KH <sub>2</sub> PO <sub>4</sub>	7.4 mM
NaCl	2.8 mM
<b>Fortification</b>	
1 M MgSO <sub>5</sub>	2 mM
50 mM CaCl <sub>2</sub>	0.10 mM
50 mg/mL thiamine	0.12 mM
50 mg/mL biotin	0.16 mM
20 % glucose	0.3 %
2 M ( <sup>15</sup> NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	7.6 mM
<sup>15</sup> N-ISOGRO™	N/A
<b>1000 x trace metals mixture [36]:</b>	
50 mM FeCl <sub>3</sub> in 0.12 M HCl	50 $\mu$ M
20 mM CaCl <sub>2</sub>	20 $\mu$ M
10 mM MnCl <sub>2</sub>	10 $\mu$ M
10 mM ZnSO <sub>4</sub>	10 $\mu$ M
2 mM CoCl <sub>2</sub>	2 $\mu$ M
2 mM CuSO <sub>4</sub>	2 $\mu$ M
2 mM NiCl <sub>2</sub>	2 $\mu$ M
2 mM Na <sub>2</sub> MoO <sub>4</sub>	2 $\mu$ M
2 mM Na <sub>2</sub> SeO <sub>3</sub>	2 $\mu$ M
2 mM H <sub>3</sub> BO <sub>3</sub>	2 $\mu$ M

## ISOLATION, FOLDING AND PURIFICATION

The insoluble His-KSI-HBD2 fusion was extracted from inclusion bodies by a series of extraction steps as previously described [35]. Briefly, the cell pellet was suspended in buffer 1 (50 mM Tris, 25% sucrose, 1 mM EDTA, 0.1% sodium azide, 10 mM DTT, pH 8.0) followed by the addition of lysozyme, MgCl<sub>2</sub> and DNase. To this was added buffer 2 (50 mM Tris, 1% Triton X-100, 100 mM NaCl, 0.1% sodium azide, 10 mM DTT, pH 8.0) and EDTA. The sample was frozen in liquid N<sub>2</sub> and immediately thawed at 37 °C. To this extract,

MgCl<sub>2</sub> was added, incubated at room temperature and then centrifuged. The pellet was resuspended in buffer 3 (buffer 1 without the sucrose but with the addition of 100 mM NaCl and 1 % Triton X-100), sonicated, then centrifuged. The resulting pellet was then washed with buffer 4 (buffer 3 with 1 M GuHCl), sonicated with 4 x 30 second bursts and centrifuged. The fusion protein was dialyzed against Tris buffer at pH 8.0 resulting in precipitation which was recovered by centrifugation. To cleave the HBD2 from the His-KSI fusion, the insoluble protein was resuspended in a Tris buffer containing 6 M GuHCl and acidified with HCl (pH ~1.0) before addition of 0.25 M CNBr. The reaction was dialyzed against Tris buffer at pH 8.0 to precipitate the His-KSI. The precipitate was removed by centrifugation and the supernatant containing HBD2 was purified by RP-HPLC on a Jupiter™ Proteo column (Phenomenex). A gradient with increasing acetonitrile (5 to 55 % in 50 minutes) was employed. The partially reduced peaks were collected, lyophilized and the reduced in 20 mM TCEP for 3 hours. The completely reduced peptide was then purified and lyophilized (3 mg). For oxidation, the reduced peptide was dissolved in 0.8 M GuHCl, 0.01 M NaHCO<sub>3</sub>, 0.3 mM cysteine, 0.003 mM cystine/HCl and stirred under nitrogen for 4 hours. The reaction was purified using RP-HPLC as described above and HBD2-containing fractions were lyophilized. A white solid with a mass corresponding to the fully oxidized form was obtained (1.5 mg of oxidized <sup>15</sup>N HBD2 from 7 g of wet paste).

## MASS SPECTROMETRY

Characterization of the recombinant <sup>15</sup>N HBD2 peptide was performed using the accurate mass capabilities of a 9.4 T Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA). Nano-ESI was performed using a TriVersa Nanomate running in infusion mode and operated in the positive mode. Prior to analysis, peptides were reconstituted to a final concentration of 5 μM in 50:49:1 MeOH: water: formic acid. The predicted isotopic abundance was determined using published methods within the XMASS and DataAnalysis software (Bruker) [37].

## NMR SPECTROSCOPY

0.5 mg of <sup>15</sup>N labelled HBD2 was dissolved in 0.45 mL 20 mM sodium acetate buffer and 0.05 mL D<sub>2</sub>O to a final concentration of 0.23 mM and final pH of 4.7. All NMR spectra were recorded on a 600 MHz Bruker AVANCE instrument equipped with a cryoprobe and operating at 25 °C. For 1D spectra, 128 scans were taken with a spectral width of 15 ppm and 8000 complex points. Double pulsed field gradient spin echo was used for water suppression [38]. For the <sup>1</sup>H-<sup>15</sup>N HSQC experiment, 8 scans were taken for each increment, using acquisition times of 125 ms to sample both *F*<sub>1</sub> and *F*<sub>2</sub> dimensions.

For both the 3D HSQC-TOCSY and 3D HSQC-NOESY, spectral widths of 11, 17 and 17 ppm were used in <sup>1</sup>H (indirect), <sup>15</sup>N and <sup>1</sup>H (direct) dimensions, respectively. WATERGATE suppression was used for both

experiments [39]. For both spectra, 192 and 2048 complex points were acquired in  $F_1$  and  $F_3$ , respectively. In  $F_2$ , 48 and 40 complex points were collected for the TOCSY and NOESY experiments, respectively. The relaxation delay was set to 1.3 sec for both and the mixing time was 70 ms for the TOCSY and 150 ms for the NOESY. The NMR data was processed using Azara, v2.7 [40] and the cross peaks were assigned using CcpNmr Analysis [41].

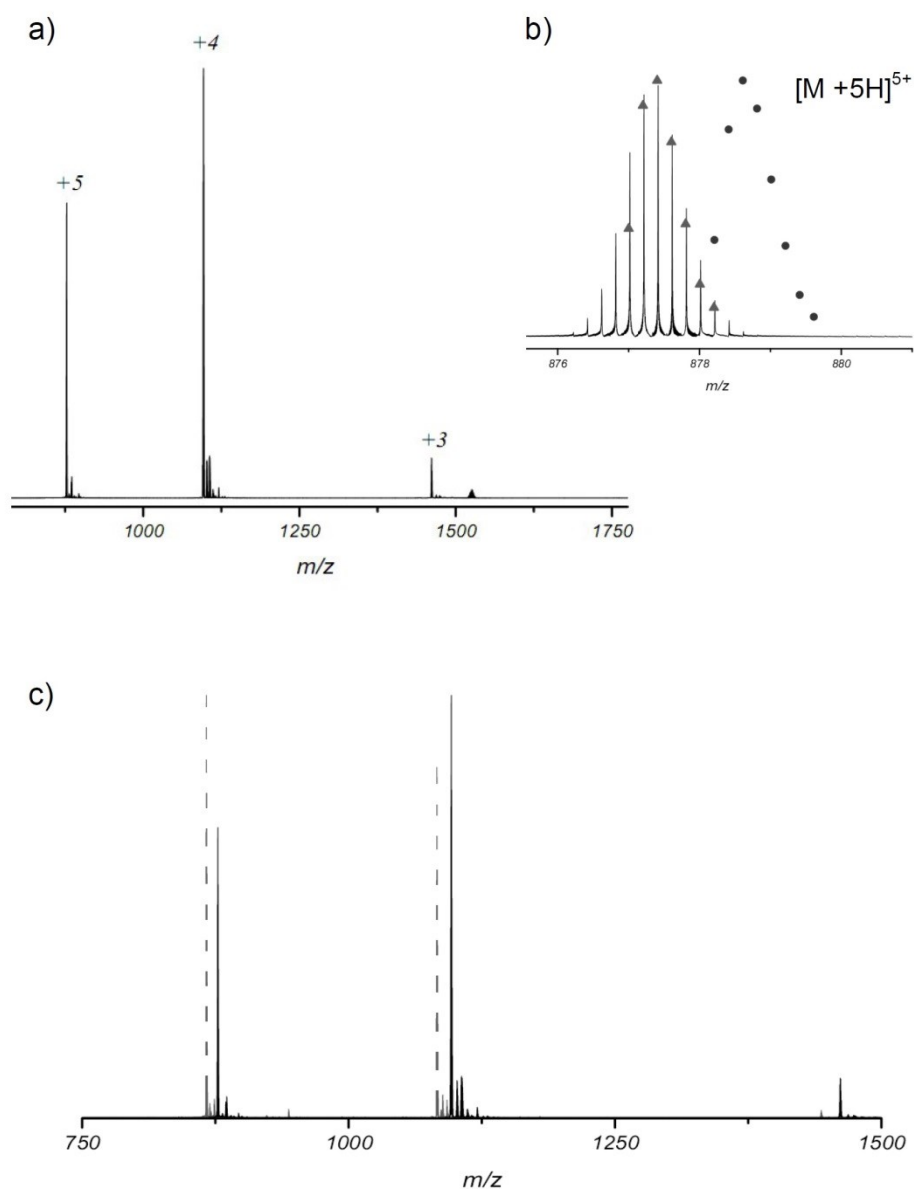
## Results and discussion

We report the preparation of the first isotopically labelled defensin,  $^{15}\text{N}$  human  $\beta$ - defensin 2 ( $^{15}\text{N}$  HBD2). It was found that *E. coli* grown in ISOGRO™ with trace metal additives significantly increased the expression of the isotopically labelled KSI-HBD2 fusion, reduced the growth and expression time and ultimately improved the yield of the isolated peptide. After the fusion protein was cleaved by CNBr and the insoluble fraction was removed from the defensin, the peptide was purified and folded using a cysteine/cystine redox buffer. The retention time of the peptide matched that of the non-labelled HBD2 confirming the isolation of  $^{15}\text{N}$  HBD2 with  $\beta$ -connectivity (Cys<sup>1-5</sup>, Cys<sup>2-4</sup>, Cys<sup>3-6</sup>, data not shown) [35].

The FT-ICR mass spectrum of  $^{15}\text{N}$  labelled HBD2 (Figure 1a) shows an ion envelope containing three charge states ( $[\text{M}+5\text{H}]^{5+}$ ,  $[\text{M}+4\text{H}]^{4+}$ ,  $[\text{M}+3\text{H}]^{3+}$ ) and upon deconvolution, gives the average mass of the oxidized  $^{15}\text{N}$  HBD2 of 4382.5 Da. The oxidized and reduced forms of  $^{15}\text{N}$  HBD2 have molecular formulas of  $\text{C}_{188} \text{H}_{316} \text{O}_{50} \text{S}_6^{15}\text{N}_{55}$  and  $\text{C}_{188} \text{H}_{310} \text{O}_{50} \text{S}_6^{15}\text{N}_{55}$ , respectively. High resolution of the +5 charge state (Figure 1b) reveals the peptide was fully oxidized with three disulfide bonds shown by an excellent fit between the predicted and experimental isotope abundances. Figure 1c shows the mass spectra of the unlabelled and labelled HBD2 which differ in mass by 55 Da. These data confirm 100 % incorporation of the  $^{15}\text{N}$  isotope accounting for all 55 nitrogen atoms of HBD2.

Previously, the NMR structure of unlabelled HBD2 was obtained in phosphate buffers at both pH 3.7 and 6.4 [22]. Although pH 3.7 is outside the buffering range of the phosphate buffer (pH 6-13), the data collected at this pH resulted in spectra with better resolution compared to data collected at pH 6.4 [22]. Therefore, to solve the solution structure of the unlabelled HBD2, the NMR spectra were obtained at pH 3.7. For the work presented in this paper, an acetate buffer was used because the desired acidic pH was within its buffering range (pH 3.8 to 5.8). Furthermore, data collected in this buffer at pH 4.7 gave spectra with further improved spectral resolution.

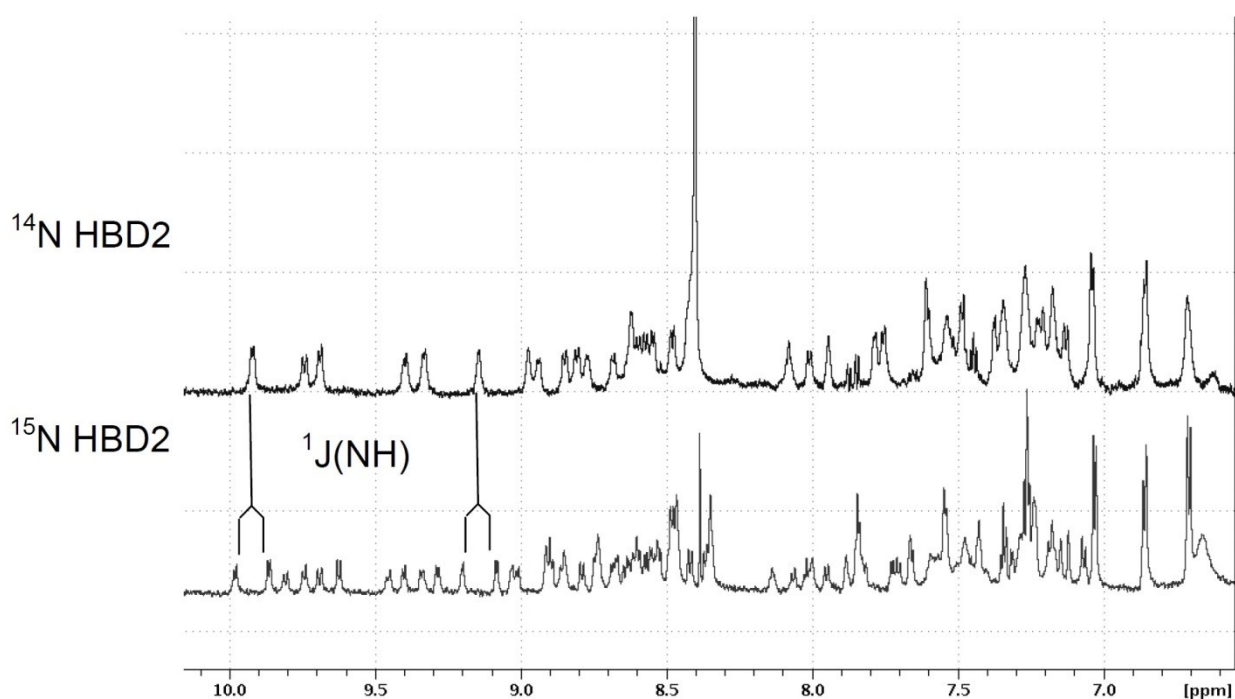




**Figure 1.** **a)** FT-ICR mass spectrum of 5  $\mu\text{M}$   $^{15}\text{N}$  labelled HBD2 in 50:49:1 MeOH: water: formic acid showing the  $[\text{M}+5\text{H}]^{5+}$ ,  $[\text{M}+4\text{H}]^{4+}$ ,  $[\text{M}+3\text{H}]^{3+}$  charge states. **b)** High resolution mass spectrum of the +5 charge state. Overlaid on the experimental mass spectra data are the theoretical isotopic envelopes for the  $^{15}\text{N}$  HBD2 ( $\blacktriangle$  oxidized form and  $\bullet$  reduced form). **c)** The mass spectra of  $^{14}\text{N}$  HBD2 (dashed line) versus  $^{15}\text{N}$  HBD2 (solid line).

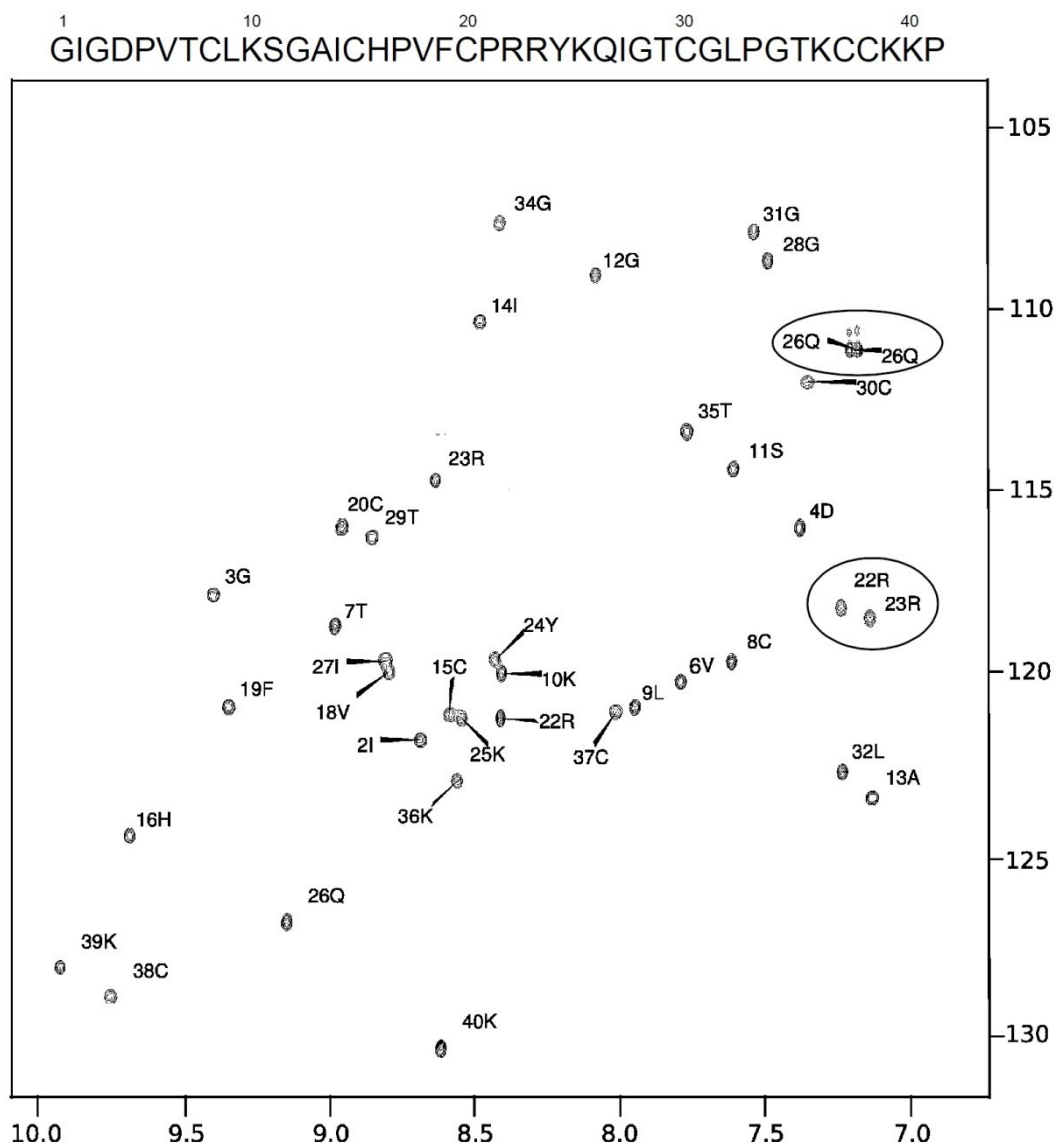
Figure 2 shows the amide region of the  $^1\text{D}$   $^1\text{H}$  NMR spectra of  $^{14}\text{N}$  and  $^{15}\text{N}$  HBD2. The sharp, well-dispersed peaks are indicative of a defined folded structure as compared to the poorly resolved spectra of linear  $\beta$ -

defensins [29]. The chemical shifts of the amide protons in the  $^{15}\text{N}$  labelled sample remain the same compared to the unlabelled peptide, but each resonance peak splits into a doublet in the spectrum of the  $^{15}\text{N}$  HBD2 due to the  $^1J(^{15}\text{NH})$  coupling. The peaks at and below 7 ppm that do not show this splitting belong to the aromatic protons of Phe 19 and Tyr 24.



**Figure 2.** The amide region of the 600 MHz 1D  $^1\text{H}$  NMR spectra of  $^{14}\text{N}$  and  $^{15}\text{N}$  HBD2 in 20 mM acetate buffer at pH 4.7 and 25°C.

The 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of  $^{15}\text{N}$  HBD2 in acetate buffer at pH 4.7 is shown in Figure 3. The amide resonances were assigned using 3D HSQC-TOCSY and 3D HSQC-NOESY [42] spectra. The data used to solve the NMR structure of unlabelled HBD2 contained several overlapping amide proton chemical shifts (Biological Magnetic Resonance Data Bank, PDB file 1fqj) [22]. The main advantage of the approach presented here is that the incorporation of the isotopic label allows for all the amide resonances to be fully resolved by a 2D NMR experiment. In addition, cross peaks due to the side chain  $\gamma$ -NH groups of Arg and Gln are observable. A complete assignment of the amide resonance peaks is included in the Supplementary Material.



**Figure 3.** 600 MHz 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of 0.23 mM  $^{15}\text{N}$  labelled HBD2 in 20 mM sodium acetate buffer at pH 4.7 and 25 oC. The sequence is given above the spectrum. The side chain amide cross peaks of residues 22R, 23R and 26Q are circled.

The 3D NOESY strips in the amide regions and the amide/ $\alpha$  proton regions from Val 6 to Ala 13 are shown in the Supplementary Material (Figures S1 and S2). An  $\alpha$ -helix region generally contains  $d_{\text{NN}}(i, i+1)$  connectivities and relatively weak  $d_{\text{aN}}(i, i+1)$  connectivities, whereas  $\beta$ -sheet regions are characterized by very intense  $d_{\text{aN}}(i, i+1)$  connectivities [42]. The presence of  $\text{NH}_i\text{-NH}_{i+1}$  cross peaks for Val 6 to Ala 13 supports the  $\alpha$ -helical secondary structure, which is similar but more extended than those previously reported by x-ray (Pro 5 to Lys 10) [21] and NMR spectroscopy (Val 6/Thr 7 to Leu 9/Lys 10) [22]. The low intensity or lack of cross peaks in Figure S2 is also consistent with the  $\alpha$ -helical secondary structure. Figure S3 shows

strong  $\alpha\text{H}_i\text{-NH}_{i+1}$  cross peaks between neighbouring residues indicative of the  $\beta$ -strand secondary structure from Thr 35 to Lys 40 which agrees with the previously solved structures of HBD2. The excellent dispersion and signal-to-noise of the HSQC spectrum revealed that the recombinant  $^{15}\text{N}$  HBD2 peptide is an ideal candidate for binding studies with a range of biologically-relevant ligands by NMR spectroscopy (Seo et al, in preparation).

## Conclusion

Here, the preparation of the first isotopically labelled  $\beta$ -defensin is presented along with the fully assigned 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum. The combination of  $^{15}\text{N}$  NISOGRO™ with trace metals in the growth medium substantially increased the efficiency of protein expression in *E. coli*. This optimized method yielded  $^{15}\text{N}$  HBD2 peptide with complete incorporation of the isotopic label as confirmed by high resolution mass spectrometry. Previously, the solution structure of unlabelled HBD2 was determined by two groups [20, 22] and provide an excellent basis for the future interaction studies of this labelled  $\beta$ -defensin at an atomic level. However, overlapping resonances in the amide region of the unlabelled sample prevented unambiguous identification of individual residues which would be further exasperated when studying the interactions of HBD2 with other molecules. The use of an isotopically labelled peptide allowed for multidimensional NMR experiments to be performed, which facilitated the assignment of all the amide resonances of  $^{15}\text{N}$  HBD2. A well-resolved, fully-assigned HSQC spectrum provides a straightforward detection of the binding of HBD2 with various ligands. Such research is important for understanding the structure-function relationship of  $\beta$ -defensins. The interaction studies using  $^{15}\text{N}$  labelled human  $\beta$ -defensin 2 are currently underway in our laboratory.

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